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Minimal Requirement for a Lentivirus Vector Based on Human Immunodeficiency Virus Type 1

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The use of human immunodeficiency virus vectors for gene therapy is hampered by concern over their safety. This concern might be ameliorated, in part, if the viral accessory genes and proteins could be eliminated from the vector genomes and particles. Here we describe a minimal vector system that is capable of transducing nondividing cells and which does not contain *tat*, *vif*, *vpr*, *vpu*, and *nef*.

To date, murine leukemia virus (MLV)-based retroviral vectors have been most frequently used for gene therapy. This is because of their efficient transfer, stable integration, and relatively long-term expression of foreign genes (43). However, one major drawback of these vectors is their inability to transduce mitotically inactive cells. Many types of cells that are attractive targets for research or clinical therapy do not divide or divide slowly. However, one subclass of retroviruses, the family *Lentiviridae*, can infect nondividing cells. This property makes these viruses, including human immunodeficiency virus (HIV), attractive for gene transfer into nondividing cells.

A number of efforts have been made to develop HIV type 1 (HIV-1)-based packaging systems following early studies to define the sequences required for packaging (25). A simple, replication-defective vector based on HIV-1 was first constructed and used for analysis of virus infectivity by Page et al. (35), and transfer of the foreign genes into a CD4$^+$ T-cell line by a HIV-1-based vector was demonstrated (38). Other groups have designed HIV-1-based vectors that are Tat inducible (9) or that use heterologous internal promoters (46). Efforts to establish a stable producer cell line have also been made (13, 37, 57). The viral titers obtained with these vectors are generally low (10^2 to 10^4 infectious particles per ml), although some improvements came with pseudotyping of the vector particles with vesicular stomatitis virus glycoprotein (VSV-G). Pseudotyped vectors can be concentrated by simple ultracentrifugation without significant loss of infectivity (3, 39). Other advantages of pseudotyping with VSV-G are a broad host range and elimination of homologous recombination to generate replication-competent viruses. By use of this pseudotyped system, transduction of nondividing neuronal cells in vivo has been demonstrated, including sustained long-term gene expression in adult rat brains (33, 34). Taken together, these observations illustrate the promise of HIV vectors for use in gene therapy.

The remaining question is safety. To create a safe, replication-defective retroviral vector, viral components (*gag-pol*, *env*, and the vector genome) must be segregated onto three separate plasmids and the sequence overlap between them must be minimized. These tasks have been successfully achieved with no replication-competent virus detected (33, 40). Another concern about lentiviral vectors is that they are distinct from oncoretrovirus-based vectors in that they possess auxiliary genes in addition to the three common retroviral genes *gag*, *pol*, and *env*. Our relative ignorance of the functions of the products of these accessory genes makes them significant factors in considerations of the safety of lentiviral vectors. All of the HIV vector systems previously reported contain some or all of the accessory genes. HIV-1 has six such genes, *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* (51, 54). Some of these have been associated with possible pathologies. For example, HIV-1 Tat has been implicated in the development of Kaposi's sarcoma (4, 5, 16). HIV-1 Vpr causes cell cycle arrest and apoptosis, and it has been suggested that this is the cause of T-cell dysfunction in AIDS patients (23). Also, extracellular Vpr present in peripheral blood has been suggested to contribute to tissue-specific pathologies associated with HIV infection since Vpr induces cell proliferation and differentiation (26, 27).

A safe and efficient vector system would exclude any nonessential viral proteins which may be present in the viral stock and which may have deleterious effects. It is therefore desirable to determine the requirement of each auxiliary gene for virus production, transduction, and integration and to eliminate any unnecessary genes from the system. In this study, we have constructed a minimal vector system which does not contain *tat*, *vif*, *vpr*, *vpu*, and *nef*. The only remaining auxiliary gene is therefore *rev*, which, with RRE, is required for efficient RNA handling in this system.

Vector production system. HIV-1-based vectors were designed to be produced from transient three-plasmid cotransfection into 293T cells (Fig. 1). The vector genome, the HIV-1 *gag-pol* gene, and the VSV-G gene were placed on three separate plasmids. This packaging system lacks the accessory genes *nef*, *vpu*, and *vpr* and has the potential to eliminate *tat*, *rev*, and *vif* (see later).

Virus was generated by calcium phosphate transfection of 293T cells and used for transduction as previously described (12) but with the following modifications. After incubation of the cells on 60-mm-diameter dishes with DNA-calcium phosphate precipitates for 12 h, the medium was replaced with 2.5 ml of fresh medium and incubated for 36 h and then the supernatant was used for transduction in the presence of Polybrene (8 μg/μl). No replication-competent virus from the packaging system was detected after 51 days of culturing (11 passages).

Tat independence. The first distinct property of our vector system is that Tat is not expressed from the packaging components, the *gag-pol* expression plasmids and pRV67 (Fig. 1).

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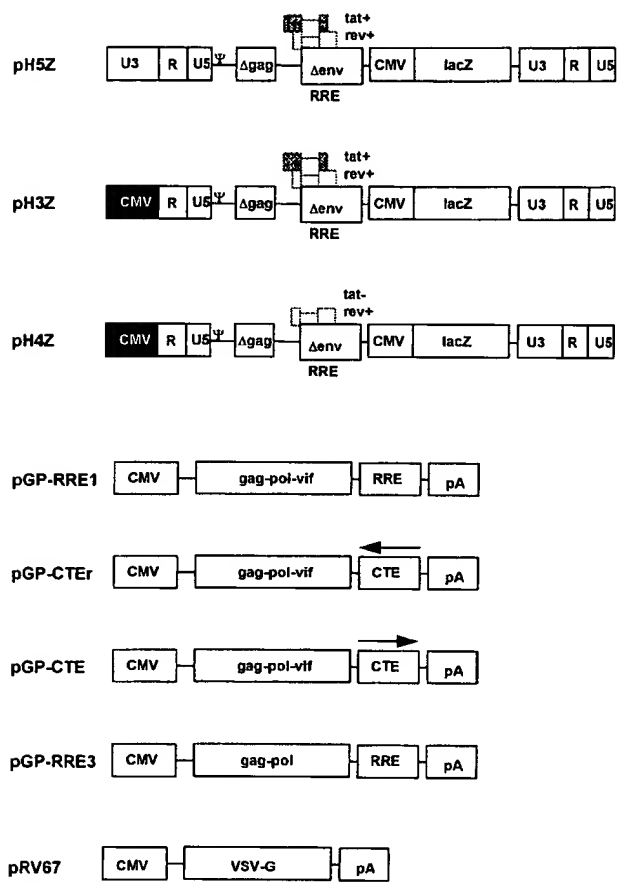

FIG. 1. Basic components of the packaging system. Vector genome plasmids pH5Z, pH3Z, and pH4Z were derived from pWI3 (24) and inserted into pBluescript KS+ (Stratagene). They have a number of structural features in common. To achieve efficient packaging by HIV cores, the vectors contain the first 778 nt of *gag* (36). A frameshift mutation created by filling in at the *Cla*I site (HIV-1 HXB2 coordinate 830 [GenBank accession no. M28248]) prevents translation of these *gag* sequences. The coordinates for HIV-1 sequences follow the Los Alamos numbering system (32). The remaining *gag-pol* sequences were removed by a deletion between *Pst*I (HXB2 nt 1415) and *Eco*RI (HXB2 nt 5743). A second deletion between *Nde*I (HXB2 nt 6402) and *Bgl*II (HXB2 nt 7620) removes part of *env*. The remaining HIV-1 sequences in the vectors include RRE and *rev* to support efficient mRNA export. The β-galactosidase reporter gene is expressed from an internal HCMV promoter. The differences among the three vector constructs, pH5Z, pH3Z, and pH4Z, are described in the text. HIV-1 *gag-pol* gene expression plasmids pGP-RRE1, pGP-CTEr, pGP-CTE, and pGP-RRE3 were constructed by first inserting the *Nar*I-*Eco*RI *gag-pol* fragment (HXB2 nt 637 to 5743) from pWI3 into pCI-neo (Promega). The *Sty*I-*Sty*I fragment containing RRE (HXB2 nt 7721 to 8053) of pWI3 was inserted downstream of the *gag-pol* coding region, resulting in pGP-RRE1 and pGP-RRE3. In the case of pGP-RRE3, a frameshift mutation in *vif* was introduced by filling in of the *Nde*I site (HXB2 nt 5122). The CTE (MPMV nt 7886 to 8373 [GenBank accession no. M12349]) was derived from an MPMV proviral clone, pSHRM15 (a kind gift from Eric Hunter), and inserted in either the reverse (pGP-CTEr) or the correct (pGP-CTE) orientation. VSV-G was expressed from the HCMV immediate-early enhancer-promoter in plasmid pRV67 (42a). CMV is the HCMV promoter, Ψ is the HIV-1 packaging signal, lacZ is the β-galactosidase-encoding gene, and pA is the polyadenylation signal. The orientations of the CTE are indicated by arrows as follows: ← for the reverse and → for the correct orientation.

HIV-1 Tat is a strong transcriptional *trans* activator and functions through a Tat activation response element located downstream of the transcription initiation site. Tat is essential for viral replication, and it is expressed from all of the previously reported production systems. However, in single-cycle infection (transduction), Tat is dispensable if the basic transcription level of the vector genome is high enough in the producer system and if any transgene is expressed from a promoter other than the HIV-1 long terminal repeat (LTR). We previously reported that a high-titer MLV stock can be produced by replacing the MLV U3 promoter with the human cytomegalovirus (HCMV) promoter (47), and so this strategy was applied to the HIV-1 vectors. Two HIV-1-based vectors, pH3Z and pH4Z, were constructed with the potent HCMV promoter (−521 to −1) by replacing U3 of the 5′ LTR (Fig. 1). pH3Z retains the *tat* coding region, while pH4Z lacks it due to a deletion (HXB2 nucleotides [nt] 5749 to 5880) encompassing the first 50 bp of the *tat* gene. These vectors were evaluated in comparison to pH5Z, which possesses the intact HIV-1 LTR structure and the *tat* gene (Table 1). The presence or absence of Tat expression from these vectors was confirmed in appropriate cotransfection studies. Each of the vector plasmids (1 μg) was cotransfected with pLTR-luc (1 μg), in which luciferase expression is Tat dependent. In the case of pH5Z and pH3Z, luciferase expression was activated 58- and 68-fold, respectively, but with pH4Z, no activation was observed (data not shown).

Supernatants from 293T cells transfected with each of the vector plasmids and pGP-RRE3 and pRV67 were assayed for transduction efficiency (Table 1). The *tat*-negative vector (pH4Z) yielded titers of $3.0 \times 10^5 \pm 0.4 \times 10^5$ *lacZ* CFU (LFU)/ml, which is comparable to those from the *tat*-positive vector (pH3Z) ($2.9 \times 10^5 \pm 0.4 \times 10^5$ LFU/ml). These titers were about 3.2 times lower than those ($9.7 \times 10^5 \pm 2.4 \times 10^5$ LFU/ml) from the HIV-1 LTR-driven, *tat*-positive vector (pH5Z). Nevertheless, this result demonstrates that, as expected, high-titer HIV vectors can be generated without the Tat *trans* activator, as long as the viral promoter is replaced with a strong constitutive promoter. Clearly, in this system, Tat is not required for any other functions in addition to its expression activation role.

Other studies have suggested a role for Tat in regulating other steps in the viral life cycle (22). For example, Harrich et al. recently, described the contribution of Tat to efficient reverse transcription (20), which appears contradictory to our result. The difference between these results and those described here might be due to differences in the virus production systems.

Requirement of Rev/RRE. Next, we considered the possibility of constructing a *rev*-independent vector system. The posttranscriptional *trans* activator Rev and its responsive element, RRE, play a role in exporting the unspliced or partially spliced

TABLE 1. Transduction efficiency of the *tat*-deficient HIV vector[a]

Vector	Promoter	Tat expression	Mean titer[b] (LFU/ml of virus stock) ± SD
—[c]	NA[d]	NA	<1
pH5Z	HIV-1 U3	+	$(9.7 \pm 2.4) \times 10^5$
pH3Z	HCMV IE[e]	+	$(2.9 \pm 0.5) \times 10^5$
pH4Z	HCMV IE	−	$(3.0 \pm 0.2) \times 10^5$

[a] Viral stocks were generated by cotransfection of pGP-RRE3 (7 μg) and pRV67 (5 μg). Results are from a representative experiment of a total of four performed.

[b] Titer was measured on 293T cells by counting the number of blue colonies following 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining 48 h after transduction.

[c] Instead of a vector genome construct, 8 μg of pCI-neo was transfected.

[d] NA, not applicable.

[e] HCMV immediate-early enhancer-promoter.

TABLE 2. Requirement of Rev/RRE for efficient vector production[a]

gag-pol plasmid	Rev expression[b]	RT activity (mU/ml)	Mean titer[c] (LFU/ml of virus stock) ± SD
pGP-RRE1	–[d]	4 ± 3	<1
pGP-RRE1	+	3,400 ± 600	$(3.1 \pm 0.0) \times 10^5$
pGP-CTEr	+	7 ± 1	$(2.0 \pm 0.2) \times 10^2$
pGP-CTE	+	95 ± 4	$(3.0 \pm 1.8) \times 10^3$

[a] Viral stocks were generated by cotransfection of each *gag-pol* expression plasmid (7 μg), pH4Z (8 μg), and pRV67 (5 μg). Results are from a representative experiment of a total of three performed.

[b] Rev is expressed from the vector genome, pH4Z.

[c] Titer was measured on 293T cells by counting the number of blue colonies following X-Gal staining 48 h after transduction.

[d] Instead of pHZ4, which is *rev* positive, the same amount of pCI-neo was transfected.

viral RNA to the cytoplasm (15, 30). The requirement of Rev in *trans* and RRE in *cis* has been shown to be partially substituted by other *cis*-acting elements (termed constitutive transport elements [CTEs]) from Mason-Pfizer monkey virus (MPMV) and related viruses (8, 58). It should be possible, therefore, to construct a Rev/RRE-independent HIV-1 vector production system by replacing HIV-1 Rev/RRE with a CTE.

Three *gag-pol* expression plasmids were analyzed: one with RRE (pGP-RRE1), one with a CTE in the reverse orientation (pGP-CTEr), and one with a CTE in the correct orientation (pGP-CTE) (Fig. 1 and Table 2). Quantitation of reverse transcriptase (RT) activity was used to measure *pol* expression (Quan-T-RT; Amersham) (Table 2). Although *pol* expression from pGP-CTE was significantly higher than that from pGP-RRE1 (without Rev) or that from pGP-CTEr, it was still only 2.8% of that from pGP-RRE1 in the presence of Rev (Table 2). The resulting viral titers from three-plasmid cotransfection using each of the *gag-pol* expression plasmids reflected the expression level of *pol* (Table 2). The highest titer, 3.1×10^5 LFU/ml, was achieved with the RRE-containing construct (pGP-RRE1). In conclusion, Rev/RRE gives maximal HIV-1 *gag-pol* expression and the substitution of Rev/RRE with an MPMV CTE was not able to provide a substantial Rev/RRE function in this context. Recently, Srinivasakumar and coworkers (49) reported a stable HIV-1 packaging system which is independent of Rev/RRE. The apparent discrepancy between these results and our own might be due to the different CTE-containing fragments used. In the system described by Srinivasakumar et al., both the MPMV CTE and its associated polyadenylation signal were used, while in our study, the polyadenylation signal was not present. Therefore, it is not clear whether the MPMV CTE, on its own, is sufficient to substitute for HIV-1 Rev/RRE. It is conceivable that the polyadenylation signal is an essential component of the RNA transport system. In addition, it is clear from other studies that CTEs function with various efficiencies, compared to Rev/RRE, depending on the assay system (8, 42, 52, 58).

Assessment of the requirements for the individual accessory genes. The HIV-1-based vector production system described above does not contain *vpr*, *vpu*, or *nef*. This suggests that these genes are not absolutely required for the function of the vector system. However, this minimal vector system provides a convenient way to examine the roles of individual accessory genes in single-cycle infection and to determine any quantitative effects they may have on transduction efficiency. Each accessory gene was expressed in addition to the basic vector components, and transduction efficiencies of dividing and nondividing cells were measured. *vif* is expressed from *gag-pol* expression plasmid pGP-RRE1 through alternative splicing, whereas pGP-RRE3 contains a frameshift mutation which abolishes the expression of functional Vif (18). The expression plasmids for *vpr* and *vpu* were constructed by inserting appropriate PCR fragments from pNL4-3 into pCI-neo to produce pCI-vpr and pCI-vpu. Similarly, Nef is expressed from the HCMV promoter in plasmid pCMV-nef (42b). Expression of each gene was verified by Western blot analysis with the appropriate antibody (data not shown). Vector preparations resulting from three- or four-plasmid cotransfections were used for transduction of dividing and nondividing cells. The transfection efficiency of each combination was similar based on β-galactosidase assay of the transfected cells (data not shown). Cells were arrested by using the DNA polymerase alpha inhibitor aphidicolin, which has been used to arrest the cell cycle in G_1/S phase to study HIV-1 infection in nondividing cells (11, 19, 28, 34). The MLV-derived vector HIT111 (47) served as a control. The transduction efficiency of the MLV-based vector was only 4 ± 3 LFU/ml on growth-arrested cells, indicating that aphidicolin treatment was working effectively (Table 3).

Vif is indispensable in a certain range of T-cell lines (i.e., CEM and H9) and peripheral blood lymphocytes but not in some T-cell lines (i.e., SupT1, C8166, and Jurkat) and other cell lines (i.e., HeLa and Cos) (17, 44, 56). The cell type used to produce Vif-defective virus determines viral infectivity, which indicates that cells that are nonrestrictive to Vif-defective virus contain a complementing host factor(s). In this system, expression of *vif* did not have an influence on viral particle production (based on RT assay of the supernatants; data not shown) or viral titer (Table 3). This result shows that Vif can be

TABLE 3. Effect of accessory gene expression on transduction efficiency[a]

Gene of interest	Expression plasmid (amt transfected [μg])	Gene expression	Mean titer[b] (LFU/ml of virus stock) ± SD: Dividing cells	Mean titer[b] (LFU/ml of virus stock) ± SD: Growth-arrested cells[c]
vif	pGP-RRE3 (7)	–	$(8.8 \pm 0.6) \times 10^5$	ND
	pGP-RRE1 (7)	+	$(8.8 \pm 0.7) \times 10^5$	$(9.2 \pm 1.7) \times 10^5$
vpr	None	–	$(3.1 \pm 1.0) \times 10^5$	$(5.1 \pm 1.3) \times 10^5$
	pCI-vpr (1)	+	$(3.0 \pm 0.2) \times 10^5$	$(3.5 \pm 0.5) \times 10^5$
	pCI-vpr (3)	+	$(3.2 \pm 0.9) \times 10^5$	$(3.6 \pm 1.1) \times 10^5$
vpu	None	–	$(4.0 \pm 0.8) \times 10^5$	$(3.2 \pm 0.5) \times 10^5$
	pCI-vpu (1)	+	$(3.7 \pm 1.1) \times 10^5$	$(3.3 \pm 1.2) \times 10^5$
	pCI-vpu (3)	+	$(3.5 \pm 0.1) \times 10^5$	$(3.0 \pm 0.0) \times 10^5$
nef	None	–	$(4.0 \pm 0.8) \times 10^5$	$(3.2 \pm 0.5) \times 10^5$
	pCMV-nef (1)	+	$(1.5 \pm 0.5) \times 10^5$	$(2.0 \pm 0.5) \times 10^5$
	pCMV-nef (3)	+	$(1.0 \pm 0.0) \times 10^5$	$(1.0 \pm 0.1) \times 10^5$
	MLV vector[d]		$(5.5 \pm 0.2) \times 10^6$	4.0 ± 3.0

[a] For *vif*, 7 μg of the *gag-pol* expression plasmid (either pGP-RRE3 or pGP-RRE1), 8 μg of pH4Z, and 5 μg of pRV67 were transfected. For the rest, 6 μg of pGP-RRE3, 7 μg of pH4Z, 4 μg of pRV67, and the indicated amount of the expression plasmid were transfected. The total amount of DNA used for transfection was kept at 20 μg by adding pCI-neo. Results are from a representative experiment of a total of at least three performed.

[b] Titer was measured on 293T cells by counting the number of blue colonies following X-Gal staining 48 h after transduction.

[c] The cells were treated with aphidicolin (15 μg/ml) for 24 h prior to transduction, and the medium was changed with fresh aphidicolin every 24 h. ND, not done.

[d] MLV vectors were generated by cotransfection of pHIT111 (5 μg), pHIT60 (5 μg), and pRV67 (5 μg).

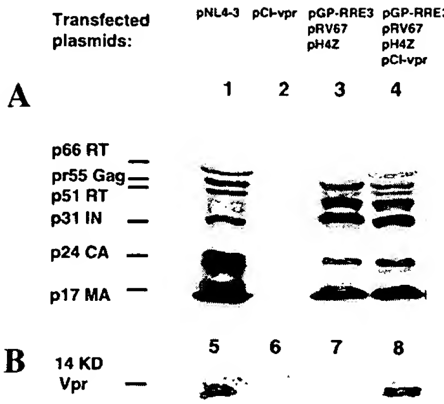

FIG. 2. Western blot analysis of viral proteins in viral particles produced by four-plasmid cotransfection. Eight micrograms of pNL4-3, 6 μg of pGP-RRE3, 7 μg of pH4Z, 4 μg of pRV67, and 3 μg of pCI-vpr were transfected, and the total amount of DNA was kept at 20 μg by addition of pCI-neo. At 48 h after transfection, viral pellets were collected from 1 ml of supernatant and separated on sodium dodecyl sulfate–10% (A) or –20% (B) polyacrylamide gels. Expression of viral proteins was visualized by using HIV-1-positive human serum (A) or rabbit anti-Vpr serum (B). Kb, kilodaltons.

excluded from the HIV packaging system when using 293T cells as producer cells.

The viral determinants that confer the ability to infect nondividing cells appear to reside in the p17 matrix (MA) protein (10, 55) and Vpr (21). Viruses with mutations in the MA protein that disrupt the nuclear localization sequence fail to replicate efficiently in nondividing cells in the absence of a functional *vpr* gene (21). Similarly, mutations in Vpr only show an apparent phenotype when the p17 nuclear localization sequence is absent. These data indicate that these viral factors function as redundant karyophilic components of the HIV-1 preintegration complex. This, in turn, suggests that Vpr would not be necessary in a vector that is to be used for the transduction of nondividing cells as long as the system contains a functional MA protein. To test this, Vpr-positive or Vpr-negative viral particles were produced by cotransfection of pCI-vpr along with plasmids pH4Z, pGP-RRE3, and pRV67. The immunoblots demonstrating *vpr* expression and incorporation are shown in Fig. 2A and B. Firstly, the Gag-Pol protein profiles of the viral particles from the vector systems are identical in the presence or absence of Vpr (lanes 3 and 4). Secondly, the amount of Vpr in the viral particles from the four-plasmid cotransfection is comparable to that from a wild-type proviral clone, pNL4-3 (lanes 5 and 8), although there are some minor differences in the profiles, perhaps due to differing processing rates. This suggests that Vpr was incorporated into viral particles efficiently. The transduction efficiencies were assayed on dividing and cell cycle-arrested cells (Table 3). As expected, the HIV-based vector transduced aphidicolin-treated 293T cells as efficiently as dividing cells whether it contained Vpr or not. Similar results were obtained with HeLa cells (data not shown).

Vpu has been shown to slightly enhance viral particle release from various cell types (45, 50, 53). To evaluate its role in this vector system, a *vpu* expression plasmid, pCI-vpu, was used in a four-plasmid strategy similar to that used for Vpr analysis. No significant increase in titer was observed, suggesting that Vpu is not necessary in this HIV-based vector system (Table 3). In agreement with this result, in a previous report (33), inclusion of *vpu* in the vector production system did not influence the titer. It is not clear why Vpu does not increase the transduction efficiency of the vectors, but it is conceivable that it is a function of using VSV-G as the envelope protein rather than HIV-1 envelope protein gp160. HIV-2 does not require Vpu activity, and HIV-2 Env can functionally replace Vpu to enhance HIV-1 core particle release (6, 7, 41). Release of particles bearing VSV-G might be similar to HIV-2 in not requiring the activity of Vpu.

Enhancement of viral infectivity by Nef has been well documented (2, 31, 48). To examine this in our vector system, the *nef* expression plasmid was cotransfected along with the three basic components of the system. Unexpectedly, the titer was three to four times lower in the presence of Nef (Table 3). However, this was only the case when VSV-G was used as the envelope protein. The enhancing effect of *nef* was clear with the HIV-1 HXB2 envelope or MLV amphotropic envelope protein (data not shown). The titer of the vector was increased 12-fold with the HIV-1 envelope protein and 2.5-fold with the MLV amphotropic envelope protein. A similar observation has been reported during the course of this study, and it was suggested that Nef functions at viral entry, which is altered by pseudotyping with VSV-G (1). For practical purposes, Nef should clearly not be included in the packaging system.

In conclusion, we have set up a minimal HIV-1-based vector production system that requires only the *rev*/RRE accessory system. It lacks *tat*, *vif*, *vpr*, *vpu*, and *nef*. The *rev*/RRE components could be removed by using a sequence such as the MPMV CTE, thereby eliminating all accessory proteins, but this does lead to a significant reduction in titer. The vector described here can transduce nondividing cells, as well as proliferating cells, with a titer of up to 8.6×10^5 LFU/ml. Furthermore, it can be concentrated easily by using ultracentrifugation with 97% recovery (data not shown). With some further refinement of the constructs, such as removal of the packaging signal present in the Gag-Pol cassette, this system should be far more acceptable as a clinical gene delivery system than previously described HIV-based vectors.

The results presented here are restricted to analyses of gene transfer in vitro. It is conceivable that the auxiliary proteins have significant effects in different tissues in vivo. However, a recent report, which substantially corroborates our findings here, showed that multiple mutations in *vif*, *vpr*, *vpu*, and *nef* did not have a significant influence on transduction of cells, including nondividing cells, in culture or in vivo (59). Only Vpr increased transduction of macrophages twofold, but this effect was seen in neither growth-arrested cells nor differentiated neurons. Our further modification that removes Tat from the system is unlikely to substantially alter the conclusions drawn from the work by Zufferey et al., given what we know of the functions of Tat. It seems likely, therefore, that lentiviral vectors without accessory genes will prove to be valuable gene therapy vectors for a range of cell types.

The system has other advantages for HIV therapy. Replacement of the HIV-1 LTR with a constitutive HCMV promoter permits the use of anti-Tat molecules such as Tat transdominant mutants (14) or Tat activation response element decoys (29) as therapeutic agents, as they do not affect vector production.

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